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CHAPTER 16

Exertional heat illness and human gene expression

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Abstract: Microarray analysis of gene expression at the level of RNA has generated new insights into the relationship between cellular responses to acute heat shock *in vitro*, exercise, and exertional heat illness. Here we discuss the systemic physiology of exertional hyperthermia and exertional heat illness, and compare the results of several recent microarray studies performed *in vitro* on human cells subjected to heat shock and *in vivo* on samples obtained from subjects performing exercise or suffering from exertional heat injury. From these comparisons, a concept of overlapping component responses emerges. Namely, some of the gene expression changes observed in peripheral blood mononuclear cells during exertional heat injury can be accounted for by normal cellular responses to heat, exercise, or both; others appear to be specific to the disease state itself. If confirmed in future studies, these component responses might provide a better understanding of adaptive and pathological responses to exercise and exercise-induced hyperthermia, help find new ways of identifying individuals at risk for exertional heat illness, and perhaps even help find rational molecular targets for therapeutic intervention.

Keywords: gene expression; exercise; heat injury; heat stroke; genomics; microarrays; peripheral blood mononuclear cells; heat shock proteins; cellular stress response

Introduction

Humans respond to environmental challenges at many levels, including behavioral, systemic, cellular, and molecular. Improvements in our ability to identify molecular alterations have led to new mechanistic insights into the effects of heat and cold on cellular and systemic function. For example, it has long been known that hyperthermia can produce changes in cellular gene expression, both *in vivo* and *in vitro*, and that some of these changes, such as increased expression of heat shock proteins (HSPs), are related to beneficial responses such as the development of thermotolerance (Lindquist, 1986; Parsell and Lindquist, 1993). More recent findings, including studies of gene expression on a large scale using microarrays, demonstrate that the gene expression response of human cells to thermal stress also includes many genes that are not traditionally designated as HSPs but that are nonetheless involved in functional pathways likely to be of physiological importance (Dinh et al. 2001; Sonna et al., 2002a, b).

It has been recognized that gene expression responses can readily be identified in human

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peripheral blood mononuclear cells (PBMCs), both *in vitro* (e.g., Sonna et al., 2002b) and *in vivo* (e.g., Sonna et al., 2004). This clinically accessible human cell type exhibits many of the responses to thermal stress that occur in other cell types traditionally used to investigate the effects of heat shock. As will be discussed in this chapter, it is increasingly evident that there are important differences in the PBMC gene expression responses that occur in response to normal physiological stresses, such as physical exercise, and those that occur during pathophysiological states, such as exertional heat illness. These differences have the potential to provide novel insights into the molecular mechanisms of exertional heat illnesses and are starting to implicate pathways not formerly associated with these diseases. We believe that a better understanding of how these pathways interact with those already known to be associated with both normal and pathophysiological responses to heat will provide the conceptual basis for the next generation of advances in the diagnosis and treatment of exertional heat illness.

This chapter will review recent insights gained from the application of microarray technologies to the study of gene expression responses to heat shock *in vitro*, and both exercise and exertional heat illness *in vivo*. We will briefly discuss the systemic physiology of exertional hyperthermia and exertional heat illness; review insights gained from *in vitro* models of human heat stress; and finally, compare the results of microarray studies of human responses to acute physical exercise and exertional heat injury.

Physiology of exertional hyperthermia and exertional heat illness

Physical exercise involves skeletal muscle contraction, which causes metabolic heat production that must be dissipated into the surrounding environment (Sawka et al., 1996; Sawka and Pandolf, 2001). During exercise, core temperature rises as a result of differences between metabolic heat production and heat dissipation. In humans, most heat dissipation occurs via transfer of heat to the surface through increases in skin blood flow and then heat loss by convection and sweat evaporation. If heat production exceeds heat dissipation, core temperature rises until dissipation matches production and a new steady state is achieved.

Compensable vs. uncompensable heat stress

Heat stress is said to be *compensable* when the mechanisms that dissipate heat are able to increase enough to quantitatively match those which produce it, resulting in an elevated steady-state core temperature (Table 1). Heat loads that exceed the capacity of heat dissipation mechanisms are said to be *uncompensable* and lead to progressive elevations of core temperature until the point of exhaustion is reached. It is important to note that the terms "compensable" and "uncompensable" refer to the core temperature response to a heat load; as will be discussed later, both compensable and uncompensable heat stress are capable of producing heat illnesses.

Table 1. Compensable vs. uncompensable heat stress

	Compensable heat stress	Uncompensable heat stress
Definition	Heat losses match heat production	Heat production exceeds heat losses
Example	Well-acclimatized and well-hydrated individual exercising in light clothing in hot weather	Individual in heavy industrial protective clothing, doing heavy work in a hot environment
Core temperature	Rises to a steady-state that is proportional to exercise intensity	Continues to rise until exhaustion
	Sustained core temperatures of $>40^{\circ}C$ are possible	Exhaustion often occurs at core temperatures $<39^{\circ}C$
Common exercise-limiting factor(s)	Hydration Fitness Energy depletion	Diversion of cardiac output to skin

The outcome of a heat stress depends on the magnitude of the heat load applied, the organism's ability to dissipate heat, and the individual's capacity to function at a higher body temperature. The environmental conditions that produce the two types of heat stress differ in important ways, as do the physiological and pathophysiological consequences thereof. During compensable heat stress, the biophysics of heat exchange permits sufficient surface heat loss by evaporative, conductive, and radiant loss mechanisms to maintain a steady-state elevation in core temperature. A typical example of this is a physically fit, well-acclimatized individual who is wearing light clothing and who is exercising under conditions of moderate heat but low humidity. Under these conditions, the core temperature achieved is roughly proportional to the exercise intensity. Core temperatures that are substantially above normal can be achieved and sustained for relatively long durations, until factors such as dehydration and energy depletion make further exertion impossible. For well-acclimatized, fit individuals subjected to compensable heat stress, function can be sustained even with core temperatures of $>40^{\circ}C$.

By contrast, during uncompensable heat stress, the biophysics of heat exchange does not allow adequate heat dissipation and core temperature continually rises during the period of exposure. An example of a setting in which uncompensable heat stress occurs is intense exercise under hot and humid conditions by an individual who is wearing heavy protective clothing. Under these conditions, skin temperature typically rises very quickly due to inadequate evaporative cooling, which in turn produces a high cutaneous blood flow at the expense of flow to other tissues. This in turn leads to diversion of blood flow from viscera and is clinically manifest as exhaustion and collapse that often occurs before the high core temperatures that are typical of compensable heat stress have been reached. For individuals exposed to uncompensable heat stress, core temperatures $<39^{\circ}C$ at the time of exhaustion or collapse can be common.

Physiological consequences of heat dissipation responses under both compensable and uncompensable conditions include ongoing loss of body water in the form of sweat and diversion of blood flow from viscera to active muscles and skin (Sawka et al., 1996; Sawka and Pandolf, 2001). This combination of reduction in circulating blood volume and redistribution of blood flow away from vital organs can have important secondary consequences. For example, ischemia of the splanchnic circulation may compromise intestinal mucosal integrity, allowing translocation of bacteria and their immuno-stimulatory products, endotoxemia, activation of innate immune systems, and the production of oxidative-nitrosative intermediates that can cause tissue damage. In addition to these perfusion-mediated problems, excessively high tissue temperatures ($>41^{\circ}C$, $105.8^{\circ}F$) can produce tissue injury directly when protective cellular mechanisms fail. There is considerable variation in the extent to which individuals can endure these consequences, and this physiological reserve can be affected by a number of identifiable factors such as degree of hydration, heat acclimatization, and aerobic fitness. Less is known about the cellular mechanisms that account for this variability in sensitivity or threshold at which exhaustion occurs. However, even in low-risk individuals, the physiological demands generated by the need for increased heat dissipation cannot be endured indefinitely. Coupled with elevated core temperature, they lead to circulatory insufficiency, cellular dysfunction, and organ injury.

The exertional heat illnesses: heat exhaustion, exertional heat injury, and exertional heat stroke

Compensable or not, any heat stress that continues beyond the individual's physiological limits of tolerance can produce illness within a spectrum of clinically related, overlapping syndromes: heat exhaustion (HE), exertional heat injury (EHI), and exertional heat stroke (EHS) (Gardner and Kark, 2001). Collectively these can be referred to as "exertional heat illnesses". Although there are no universally accepted definitions of these conditions, HE is typically defined as simple inability to continue exercise in the context of hyperthermia. EHI is characterized by evidence of end-organ injury such as elevations in serum liver enzymes but without neurological impairment beyond transient

Table 2. Examples of risk factors for heat illness

Protective against heat illness	Increased risk of heat illness
Low adiposity	High adiposity
High cardiovascular fitness	Low cardiovascular fitness
Adequate hydration	Dehydration
Prior heat acclimatization	Lack of prior heat acclimatization
	Impaired perspiration
	Antecedent febrile illness
	Previous history of heat illness
	Prior-day prodromal illness or substantial heat load
Low-intensity exercise	High-intensity exercise
Low ambient temperature and humidity	High ambient temperature and humidity
Clothing with low insulating capacity	Highly insulating or water-impermeable clothing

confusion. EHS involves significant neurological signs and symptoms, such as persistent confusion, delirium, or coma. These three conditions are best thought of as a continuum rather than separate disease processes, and it is not unusual for individuals with heat stroke to exhibit evidence of other organ injury or dysfunction.

The onset, type, and severity of heat illness are all influenced by a number of contributing factors. Common recognized clinical risk factors for the development of exertional heat illnesses are listed in Table 2. As noted, both compensable and uncompensable heat stress can produce exertional heat illness. In general terms,[1] compensable heat stress, which tends to produce marked and prolonged elevations of core temperature, is more likely to cause severe EHI and heat stroke than is uncompensable heat stress. By contrast, uncompensable heat stress is more commonly associated with HE and less severe EHI.

Historically, exertional heat illnesses were thought to occur predominantly in high risk persons such as those of low physical fitness, high body fat, unacclimatized to heat, or dehydrated (Shibolet et al., 1976; Epstein et al., 1999). It is now increasingly appreciated that exertional heat illnesses also occur in fit individuals and at lower exertional levels and heat loads than were previously considered a risk for exertional heat illness. For example, several studies have reported that many cases of exertional heat illness occur under relatively temperate conditions (Kark et al., 1996; Epstein et al., 1999; Gardner and Kark, 2001). Furthermore, the duration of exercise that precipitates decompensation need not be prolonged (<1 h in 43% of cases in one series (Epstein et al., 1999)).

There has been speculation in the literature that for some heat injury/stroke victims, a previous heat injury or illness might augment the hyperthermia of exercise, thus inducing heat illness under environmental conditions that would normally be tolerated (Montain et al., 2000). For others, antecedent viral infection or febrile illness might limit the ability of cells or tissues to adapt to heat stress and thus make them more susceptible to injury (Hasday and Singh, 2000; Sonna et al., 2004). As many as 16–18% of individuals who develop exertional heat illness also report having suffered from a nonspecific prodromal illness in the days leading up to the acute decompensation (Shibolet et al., 1967; Epstein et al., 1999). Prior-day exposure to heat stress has also been identified as a risk factor for development of exertional heat illness (Kark et al., 1996). Regardless, the incidence of exertional heat illness among individuals otherwise thought to be at low risk suggests the possibility that there may be additional, as of yet unidentified, risk factors for the development of exertional heat illness.

In summary, the risk of exertional heat illness is influenced by the magnitude and duration of hyperthermia (which in turn is determined by the balance between heat load, and dissipative

[1]This assumes that all other factors are comparable and that the individual's rise in core temperature is halted promptly on collapse (e.g., by moving to temperate conditions and achieving adequate heat dissipation).

capacity), and by the ability of cells, tissues, and organ systems to function at increased temperature. Risk factors for the development of exertional heat illness can be gleaned from epidemiological studies; some of these likely exert effects systemically (such as the insulating effects of high body fat and the limiting effects of dehydration on cardiac output). Others, such as antecedent illness, seem likely to affect the ability of cells, tissues, and organs to resist injury and continue functioning at high temperature. Thus, a comprehensive understanding of physiological and pathophysiological responses to heat must combine knowledge of systemic, organ, and tissue responses, with insights into the heat-related pathways that govern cell function.

Cellular responses to heat

Exposure to heat exerts direct effects on the constituent molecules that comprise cells. It also triggers cellular responses designed to cope with those effects, restore homeostasis, and render the cell tolerant to subsequent heat stress. These cellular responses, referred to collectively as "heat shock responses", are ubiquitous and highly conserved in eukaryotes (Lindquist, 1986; Parsell and Lindquist, 1993; Katschinski, 2004).

The effects of heat on cells include (Fig. 1): denaturation and misaggregation of proteins (Lindquist, 1986); activation of transcription factors such as heat shock factor 1 (HSF1) (Morimoto, 1998) that can lead to increased expression of stress proteins; an otherwise generalized disruption of transcription (Lindquist, 1986), RNA processing (Lindquist, 1986; Bond, 1988; Yost and Lindquist, 1991), and translation (Panniers, 1994); acceleration of enzymatic reactions (per the Arrhenius equation); changes in the activities of many signal phosphatases and kinases (Dubois and Bensaude, 1993; Han et al., 2000, 2001; Obata et al., 2000; Dorion and Landry, 2002); increased

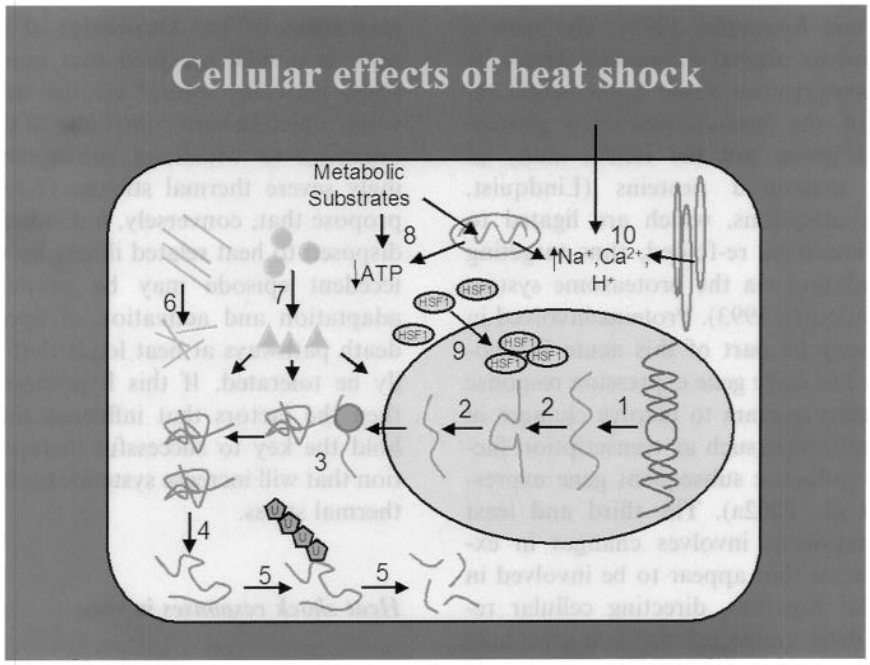

Fig. 1. Cellular effects of acute heat shock. These include effects on (1) transcription, (2) RNA processing, (3) translation, (4) protein conformation, (5) protein degradation, (6) cytoskeletal organization, (7) activities of signal transduction proteins, including stress kinases, (8) metabolism and biosynthesis, sometimes resulting in decreased cellular ATP, (9) cellular distribution and activities of critical proteins, such as transcription factor HSF-1, and (10) changes in membrane permeability leading to changes in intracellular ion concentrations.

protein degradation via the proteasomal and lysosomal pathways (Parag et al., 1987; Xu et al., 1997; Mathew and Morimoto, 1998); cell-cycle arrest (Helmbrecht et al., 2000; Kuhl and Rensing, 2000); and activation of pro- or anti-apoptotic pathways (Punyiczki and Fesus, 1998; Creagh et al., 2000; Beere, 2004). Heat can also alter membrane permeability (Weitzel et al., 1987; Gaffin et al., 1997; Skrandies et al., 1997; Koratich and Gaffin, 1999) to produce increases in intracellular sodium, hydrogen ion, and calcium concentrations, disrupt cytoskeletal components (Han et al., 2000; Dorion and Landry, 2002), and decrease intracellular stores of ATP (Findly et al., 1983; Weitzel et al., 1987).

As will be discussed in detail, the cellular response to acute heat stress itself appears to comprise at least three components. The first involves changes in the functional activities of previously translated proteins. These include activation of constitutively expressed transcription factors such as HSF1 (Morimoto, 1998), and of components of stress- and mitogen-activated protein kinase pathways (Dubois and Bensaude, 1993). The second component involves altered expression levels of proteins that comprise an acute homeostatic response. Two of the best-characterized protein families in this group are the HSPs, many of which re-fold denatured proteins (Lindquist, 1986), and the ubiquitins, which are ligated to proteins that cannot be re-folded, thus targeting them for degradation via the proteasome system (Parsell and Lindquist, 1993). Proteins involved in redox control may be part of this acute homeostatic response. The early gene expression response to heat shock also appears to involve changes in expression of molecules such as transcription factors that likely influence subsequent gene expression (Sonna et al., 2002a). The third and least understood component involves changes in expression of proteins that appear to be involved in restoring cellular function, directing cellular remodeling, and determining cellular fate after heat shock, such as regulatory proteins, proteins involved in cell-cycle control, structural proteins, and proteins involved in pro- and anti-apoptotic pathways (Sonna et al., 2002a, b). In at least some cell lines, this component also includes a number of molecules involved in cell signaling (Sonna et al., 2002a).

At the cellular level, the ultimate outcome of given heat stress appears to occur in gradations, with survival and adaptation occurring if conditions permit and if not, apoptosis in preference to necrosis (Creagh et al., 2000; Dorion and Landry, 2002). The factors that determine cellular fate after heat shock are still an active area of investigation, but it is clear that antecedent history is one of the major determinants. For example, cells that have previously been subjected to heat shock and are thus expressing high levels of HSPs can often tolerate subsequent stresses would be lethal to unconditioned cells (Lindquist, 1986; Parsell and Lindquist, 1993). In contrast, prior exposure to stimuli such as LPS, TNF-alpha, interferons, or cytokines can sensitize cells to heat shock-induced apoptosis (Buchman et al., 1993; Abello and Buchman, 1994).

We propose that a better understanding of the balance between pathways that support adaptation and survival and those that steer cells toward apoptosis and/or necrosis is critical to the advancement of our knowledge of heat-related illness. It is well accepted that non-lethal thermal stress activates several cellular adaptation pathways, which in turn contribute to the ability of the organism to withstand subsequent and increasingly severe thermal stresses (Kregel, 2002). We propose that, conversely, individuals who are predisposed to heat related illness by virtue of an antecedent episode may be prone to failure of adaptation and activation of apoptotic and cell death pathways at heat loads that would ordinarily be tolerated. If this hypothesis is confirmed, then the factors that influence this balance may hold the key to successful therapeutic manipulation that will increase systemic performance during thermal stress.

Heat shock responses in vitro

Experimentally, the best-characterized response to heat shock is increased expression of several families of HSPs (Lindquist, 1986; Parsell and Lindquist, 1993; Katschinski, 2004). In models involving tissue culture or primary isolates of cells, a very

strong heat shock response can often be elicited by exposing cells to a temperature of 5° to 6°C above the normal culture temperature for 20–60 min, followed by normothermic recovery for varying periods of time. While changes in HSP expression are often detectable during the period of hyperthermia itself, the maximal expression of HSPs commonly occurs several hours into the normothermic recovery period (e.g., Sonna et al., 2002a, b). Changes in expression have also been reported in many genes belonging to functional categories other than the 'classic' HSPs (Sonna et al., 2002a).

One of the most important mechanisms whereby heat shock alters gene expression involves activation of transcription factors, of which the best characterized is HSF1 (Morimoto, 1998; Pirkkala et al., 2001). This ubiquitous transcription factor is expressed constitutively in cytoplasmic multiprotein complexes that include HSPs and co-chaperones. When denatured protein residues accumulate in the cytoplasm as a result of heat or other protein-denaturing stresses, the HSF1 monomers are released and appear shortly thereafter in the nucleus in a trimeric, transcriptionally active form that is capable of inducing transcription of HSPs and other heat shock-responsive genes (Morimoto, 1998). Additionally, some HSPs have specialized features that permit their expression under hyperthermic conditions that globally disrupt gene expression. For example, some members of the HSP 70 family lack introns (Lindquist, 1986) and therefore can be expressed even at temperatures that disrupt RNA splicing.

Heat shock also causes changes in the activities of a number of stress-activated signal transduction pathways. These include mitogen-activated protein kinases, stress-activated protein kinases such as jun-N-terminal kinases (JNK), p38, and extracellular signal-regulated kinases (ERK) (Dubois and Bensaude, 1993; Han et al., 2000, 2001; Obata et al., 2000; Dorion and Landry, 2002). These changes modulate cellular responses to heat stress and may influence cell fate after heat shock (i.e., survival and adaptation vs. apoptosis).

HSPs serve a variety of functional roles. Many HSPs (best typified by the HSP 70 family of proteins) are chaperonins, enzymes whose primary function is to re-fold denatured proteins into a native conformation (Lindquist, 1986; Georgopoulos and Welch, 1993). Some, like members of the HSP 90 family, are also involved in the normal processing of regulatory proteins such as steroid receptors (Georgopoulos and Welch, 1993). Others are involved in regulation of cellular redox state and cellular signaling (HSP 32, better known as heme oxygenase-1 (Otterbein and Choi, 2000)), and still others are involved in targeting for degradation of proteins (the ubiquitins) (Parsell and Lindquist, 1993).

As noted, the heat shock response also involves genes other than those traditionally designated as HSPs (Sonna et al., 2002a). These include components of transcription factors (such as jun) that may produce downstream changes in gene expression. They also include several genes that likely have substantial effects on cellular function after heat shock, such as cell-cycle proteins p53 and p21 (WAF-1), signal transduction molecules such as DUSP1, and molecules involved in redox control such as Cu, Zn superoxide dismutase (Sonna et al., 2002a).

Microarray studies of the heat shock response in vitro

DNA microarray studies have confirmed the hypothesis that responses to heat shock produces extensive changes in gene expression, at least with respect to mRNA. Two recent examples of the application of DNA microarrays to the study of heat stress responses involved the study of retinal pigment epithelial cells that had survived laser burns (Dinh et al., 2001) and human PBMCs subjected to heat shock *in vitro* (43°C for 20 min followed by recovery at 37°C for 2 h and 40 min) (Sonna et al., 2002b). In both studies, extensive changes were noted in expression both of HSPs and of genes not traditionally considered to be HSPs. Changes occurred both in pathways previously known from biochemical and physiological studies to be involved in the human response to heat shock as well as in pathways whose role in the heat shock response is less well defined (Sonna et al., 2002b). Given the overlap between the changes observed in PBMCs with those typically

found in other cell lines in which heat shock responses have been studied, the identification of genes involved in cell-cycle control, gene expression, and pro- and anti-apoptotic pathways provides novel opportunities for understanding how heat stress could affect tissue function and integrated physiological responses without the need for obtaining human cells by highly invasive methods.

The heat shock response in animal models

The responses of animals exposed to hyperthermic stress has been studied extensively, and the reader is referred to excellent reviews on this topic (Moseley, 1997; Kregel, 2002) and recent work in a baboon model (Bouchama et al., 2005), mice (Leon et al., 2005, 2006), and rat (Sharma, 2006). A wide variety of tissues exhibit increased expression of HSPs in response to hyperthermic stress, and the increased expression of these proteins correlates with the development of thermotolerance. Other factors such as desensitization of the p38 and JNK pathways may also be involved in the acquisition of thermotolerance (Dorion and Landry, 2002). Expression of HSPs may also be involved in increased tolerance to non-thermal stresses such as oxidative stress and ischemia-reperfusion (Kregel, 2002) as well as in acclimation to heat (the ability to perform increasing work at high temperatures (Moseley, 1997)). The expression of HSPs within cells can therefore confer benefit at the cellular, tissue, organ, and whole organism levels. These models have the potential not only to link cellular changes to integrated physiological responses but also to ascertain the extent to which pathways other than those traditionally associated with thermal stress are involved.

Heat shock responses and moderate (febrile-range) hyperthermia

Adaptive responses induced by exercise hyperthermia may be associated with heat acclimatization (Sawka et al., 1996), the acquisition of thermotolerance, or both, as evidenced by observation that exercise-trained rats have reduced mortality when exposed to severe heat stress (Fruth and Gisolfi, 1983). Although high temperatures will elicit a maximal heat shock response (42° to 45°C for most human cell lines), recent studies also show that heat shock responses can be produced at much lower temperatures, including temperatures commonly achieved in the febrile range (38.0° to 39.5°C) (Hasday and Singh, 2000; Park et al., 2005). Temperatures in this range are commonly achieved during acute physical exercise, which raises the possibility that acute physical exercise *in vivo* might cause some of the same gene expression changes that are observed after more severe heat stress. This is conceptually important because, by comparing changes in gene expression that occur during acute physical exercise to changes in expression that occur during heat illness, it may be possible to distinguish adaptive from pathological pathways and mechanisms.

Effect of acute physical exercise on gene expression in humans

Cell types used to study gene expression responses to exercise

It is generally accepted that exercise training produces changes in gene expression in cell types such as skeletal muscle (Fluck and Hoppeler, 2003; Goldspink, 2003). Recently it has been recognized that even a single bout of exercise can produce acute changes in gene expression in skeletal muscle, at least in younger men (Jozsi et al., 2000; Mahoney et al., 2004, 2005; Bickel et al., 2005). That even a single period of exercise can induce such changes can be explained by the observation that exercising muscle is subject to a variety of stimuli that are known to alter gene expression, including elevated temperatures, hormones, neuronal activation (leading to membrane depolarization and increases in intracellular calcium), tissue hypoxia, acidosis, and mechanical deformation (Fluck and Hoppeler, 2003). Some of these factors are likely to affect not only skeletal muscle itself, but also any cells that traverse skeletal muscle capillary beds during exercise (such as circulating leukocytes). Furthermore, the exercising

muscle itself releases factors that are known to influence gene expression in other cell types. For example, several studies have reported exercise-induced release of the anti-inflammatory cytokine IL-6 (for a recent review, see Petersen and Pedersen, 2005), which in turn can induce expression of both the IL-1 receptor antagonist (also elevated in exercise, see below) and IL-10, and inhibit production of TNF-alpha (Petersen and Pedersen, 2005).

Skeletal muscle biopsy can, therefore, provide important insights into human gene expression changes that occur with exercise and hyperthermia. However, the technique is invasive, and skeletal muscle gene expression changes during exercise only yield information about the effects of exercise on that single tissue type, albeit one that is undeniably central to our understanding of the molecular physiology of exercise in humans. An understanding of normal and abnormal effects of exercise and hyperthermia on other tissue types (such as liver, bowel, neural tissue, etc.) requires animal models, the application of invasive biopsy techniques to human volunteers, or the use of representative samples of cells such as those that circulate through these tissues.

Fortunately, PBMCs, which can be readily obtained from humans, are an accessible and potentially informative cell type with which to study non-muscle responses to heat stress and exercise. PBMCs express many genes and are responsive to a wide variety of stimuli *in vitro* and *in vivo*, including heat stress. As inflammatory cells, they are centrally involved in many important systemic inflammatory responses under both physiological and pathophysiological conditions. As circulating cells, they are exposed both to systemic signals and to local signals present in perfused tissues during exercise, including temperature, pH, oxygen tension, cytokines, mechanical stresses, oxidative stress, and other local tissue factors. Indeed, there is increasing evidence that gene expression in circulating PBMCs is influenced by disease processes in isolated organs, such as pulmonary hypertension (Bull et al., 2004). Finally, studies have demonstrated convincingly that PBMCs generate a gene expression response *in vivo* to thermal stress that has remarkable fidelity to the HSP response of many other cell types (Ryan et al., 1991; Fehrenbach et al., 2000a, b, 2001; Schneider et al., 2002).

An important limitation to the use of unsorted PBMCs is that they represent a heterogeneous cell population. Measured changes in expression can, therefore, result from either actual changes in transcript level within individual cells or from changes in the distribution of PBMC subpopulations produced by a given stimulus. Although this can be of considerable utility in its own right (e.g., a stimulus that markedly alters the ratio of CD4 to CD8 lymphocytes would likely produce measurable changes in the gene expression profile of peripherally obtained PBMCs that could be used for diagnostic purposes), it is an inherent limitation of the use of PBMCs that must be kept in mind when evaluating the gene expression literature.

Microarray studies of exercise-induced gene expression changes in PBMCs

Two important microarray studies have recently shed light on the responses of PBMCs to acute physical exercise.

Connolly et al. (2004) studied the effect of acute physical exercise on PBMC gene expression in 15 moderately fit (VO_2 peak, 35–45 ml/min/kg) young men aged 18–30. The exercise protocol consisted of cycling at ~80% of peak VO_2 for 30 min. They did not report body temperature measurements, but such exercise intensities would be expected to increase core temperatures to ~39°C (Sawka et al., 1996). Samples were obtained for microarray analysis before exercise, immediately after exercise, and after 60 min of post-exercise recovery. RNA samples were analyzed on a single-dye platform containing 22,283 sequences (Affymetrix HU133A). Moderately strict criteria were used to call a difference in expression statistically significant (estimated false positives $\leq 1\%$).

In this study, the subjects demonstrated a rise in serum lactate and growth hormone that peaked at the end of exercise, as well as a rise in IL-6 and IL-1ra that peaked at the end of the recovery period. An increase in circulating leukocytes was noted at the end of exercise in all three major lineages (granulocytes, lymphocytes, and monocytes),

which returned to baseline values at the recovery time point. The array data showed a substantial, time-dependent gene expression response to exercise involving several hundred genes. Interestingly, increases in gene expression dominated the expression pattern at the end of exercise, whereas both increases and decreases in gene expression were evident at the recovery time point. In all, these authors reported that 311 genes demonstrated significant expression changes during exercise (post- vs. pre-exercise) and 292 genes demonstrated expression changes after recovery as compared to the pre-exercise baseline (recovery vs. pre-exercise). A total of 552 genes showed differences in expression between the post-exercise and the recovery time points.

The genes affected by exercise (at one or both of the time points studied) included many "classic" HSPs, some well-known non-specific stress proteins (such as DUSP1), and several inflammatory modulators. Changes in IL-6 mRNA were not detected, consistent with prior literature concluding that skeletal muscle, not PBMCs, is the predominant source of the circulating IL-6 produced during exercise (Petersen and Pedersen, 2005). Additionally, many of the affected genes are involved in cell growth, proliferation, and differentiation as well as in transcription and signal transduction.

The breadth of the gene expression response to acute physical exercise was similar to observations made in heat shock microarray studies of PBMCs *in vitro* (Sonna et al., 2002b) and prompted us to perform a more detailed comparison of the specific genes affected by the two stresses. In the published supplement to their report, Connolly et al. (2004) listed 433 specific sequences that were affected by exercise in one or more of their paired comparisons. From this list, 345 corresponding sequences were identified on the arrays used to perform the previous *in vitro* heat shock experiments (the U95A)[2] (Sonna et al., 2002b). We queried this list to identify genes that displayed a statistically significant change in expression as a result of *in vitro* heat shock. To maximize methodological comparability with the data of Connolly et al., we performed our query without the post-hoc filters used in the previous *in vitro* study (Sonna et al., 2002b), such as expression calls and arbitrary fold-change cutoffs.

Table 3 provides a comparison of gene expression from acute physical exercise and *in vitro* heat shock. Of the 345 sequences affected by exercise for which cross-platform comparisons could be made, 184 sequences were different from baseline at the end of exercise (168 increased and 16 decreased) and 104 were different from baseline after recovery (61 increased and 43 decreased). Approximately 1/5 of these genes showed similar changes after heat shock *in vitro* (Table 3). This overlap is somewhat larger than that found (11–14%) in a control comparison of the effects of acute physical exercise in PBMCs to the effects of hypoxia on a different cell line (exposure of HepG2 hepatocytes in culture to 1% oxygen for 24 h (Sonna et al., 2003)). Furthermore, the degree of overlap increased substantially when the analysis was limited to genes considered to be of high interest by Connolly et al., that were further classified as "stress proteins and HSPs". Of 15 sequences for which cross-platform comparisons could be made (representing 11 genes of this class)[2], all showed increased expression at one or both of the time points after exercise as compared to baseline and of these, 12 (80%) were also increased significantly by heat shock *in vitro*. The three sequences that showed responses after heat shock that differed from acute physical exercise corresponded to dual specificity phosphatase 5, HIF1A (both unaffected by heat shock *in vitro*) and HIF-1 responsive RTP801 (decreased by heat shock).

Among the "high interest" genes identified by Connolly et al., the degree of overlap between the effects of exercise and the *in vitro* PBMC heat shock responses reported by Sonna et al. (2002b) was also greater for stress proteins and HSPs than for other categories. For inflammatory response genes, the overlap with heat shock responses was 17 and 31% at end-exercise and recovery, respectively. For genes classified as growth and

[2]In some cases, direct correspondences were redundant (meaning, more than one sequence could be found on one array that corresponded to the targeted sequence on the other). In others, correspondence was indirect (i.e., corresponding sequences were identified on the two platforms that targeted the same gene though not necessarily not the same target sequence).

Table 3. Overlap between the effects on gene expression of acute exercise *in vivo* (Connolly et al., 2004; Zieker et al., 2005) and heat shock *in vitro* (Sonna et al., 2002b)

Study	Time point	Category	Direction of change		
			Increased	Decreased	Either
Connolly et al. (PBMCs)	Post-exercise	Total sequences affected[a]	168 (100%)	16 (100%)	184 (100%)
		Sequences similarly affected by heat shock	34 (20%)	7 (44%)	41 (22%)
		Sequences differently affected by heat shock[b]	134 (80%)	9 (56%)	143 (78%)
	Recovery	Total sequences affected[a]	61 (100%)	43 (100%)	104 (100%)
		Sequences similarly affected by heat shock	15 (25%)	6 (14%)	21 (20%)
		Sequences differently affected by heat shock[b]	46 (75%)	37 (86%)	83 (80%)
Zieker et al. (Whole blood)	Post-exercise	Total sequences affected[a,*]	13 (100%)	22 (100%)	35 (100%)
		Sequences similarly affected by heat shock	4 (31%)	8 (36%)	12 (34%)
		Sequences differently affected by heat shock[b]	9 (69%)	14 (64%)	23 (66%)

[a]Relative to pre-exercise baseline.
[b]Includes sequences not significantly affected by heat shock and sequences that showed a statistically significant change of opposite direction.
*Includes all differentially-expressed sequences regardless of Bonferroni-adjusted *P* value.

transcription factors, the overlap was 19 and 8%. Specific examples of genes that display overlap in the responses to heat shock and acute physical exercise are listed in Table 4.

In summary, a portion of the *in vivo* PBMC gene expression response to physical exercise appears to involve changes that are also produced by heat shock *in vitro*, particularly among stress proteins and HSPs. These observations are consistent with the hypothesis that physical exercise is a complex stimulus in which the accompanying hyperthermia might account for at least some of the observed changes in PBMC gene expression.

Zieker et al. (2005) studied trained runners (peak VO_2 not reported but average weekly distance run was 52.2 km (32.4 miles) and all had been training for at least 2 years), aged 28–58, who were competing in a half-marathon on a cool and humid day (ambient temperature 1°C or 33.8°F) on hilly terrain. The average race time was 105 min (range 77–139 min). Samples were drawn for microarray analysis before, immediately after, and 24 h after the race. In this study, RNA was extracted from whole blood, not simply PBMCs. Samples were analyzed using a custom dual-dye spotted array platform containing 277 probes primarily focused on genes involved in inflammatory responses. Findings of interest were confirmed by real-time PCR. As in the study by Connolly et al. (2004), an increase in circulating leukocyte count had occurred at the end of exercise that was principally attributable to an increase granulocytes. The number of monocytes also increased but there was a decrease in lymphocytes. These changes returned to baseline by the recovery time point.

The microarray array data demonstrated that ~10% of the genes (29 sequences representing 28 genes) showed expression differences after the race as compared to baseline. The magnitudes of the changes reported were generally small, with only three genes showing increases of twofold or greater (selectin L, thioredoxin, IL 8 receptor alpha) and only four showing decreases of twofold or greater (CD81, ICAM2, Chemokine receptor 1 and CD2). Furthermore, although all 29 sequences showed changes that could be considered statistically significant by unadjusted *P* values, only seven met the very stringent criteria for statistical significance that the authors applied to minimize false positive reports (Bonferroni adjustment).[3] Importantly, the

[3]The affected sequences corresponded to: selectin L (upregulated), CD81, CD244, integrin alpha X, glutathione S-transferase M3, ICAM2, and chemokine receptor 1 (all downregulated). To account for multiple comparisons, the authors used a factor of 345 to adjust *P* values (Zieker, personal communication). Accordingly, we included all 29 genes reported by the authors as having Bonferroni-adjusted $P<1$ (i.e., unadjusted $P<0.003$) in our comparative analysis. This broader list of 29 genes included the IL-1 receptor antagonist, which had a Bonferroni-adjusted *P* value of 0.93 on the microarray but which the authors found on real-time PCR to be upregulated in this study, in keeping with observations made by others.

Table 4. Examples of genes that are similarly affected in PBMCs by acute physical exercise and heat shock

Stress response genes and HSPs	
Increased	
DUSP1	Dual-specificity phosphatase 1
DUSP2	Dual-specificity phosphatase 2
DUSP3	Dual-specificity phosphatase 3
HSPB1	Heat shock 27 kDa protein 1
DNAJA1	DnaJ (Hsp 40) homolog, subfamily A, member 1
DNAJB1	DnaJ (Hsp 40) homolog, subfamily B, member 1
HSPA1A	Heat shock 70 kDa protein 1A
HSPA1B	Heat shock 70 kDa protein 1B
HSPCA	Heat shock 90 kDa protein 1, alpha
HSPH1	Heat shock protein 105
STIP1	Stress-induced phosphoprotein 1 (HSP 70/HSP 90 organizing protein)
Growth factors and transcription	
Increased	
EGR1	Early growth response 1
NCOA1	Nuclear receptor co-activator 1
NR4A2	Nuclear receptor subfamily 4, group A, member 2
RUNX3	Runt-related transcription factor 3
S100A9	S100 calcium binding protein A9 (calgranulin B)
TCF8	Transcription factor 8
TIEG	TGFB inducible early growth response
YES1	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1
Inflammatory and immune response genes	
Increased	
CSF3R	Colony stimulating factor 3 receptor (granulocyte)
NCR3	Lymphocyte antigen 117
PTPN22	Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)
TNFSF14	Tumor necrosis factor (ligand) superfamily, member 14
XCL2	Chemokine (C-motif) ligand 2
Decreased	
IGHM	Immunoglobulin heavy constant mu
NCF1	Neutrophil cytosolic factor 1 (47 kDa, chronic granulomatous disease, autosomal 1)
TNFRSF12	Tumor necrosis factor receptor superfamily, member 12 (translocating chain association membrane protein)
Metabolism and biosynthesis	
Increased	
BPGM	2,3-bisphosphoglycerate mutase
NDUFB7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7, 18 kDa
Other	
Increased	
MADH7	MAD, mothers against decapentaplegic homolog 7 (*Drosophila*)
SNTB2	Syntrophin, beta 2 (dystrophinassociated protein A1, 59 kDa, basic component 2)
STK39	Serine threonine kinase 39 (STE20/SPS1 homolog, yeast)

authors confirmed several of the observed gene expression changes by real-time PCR, including some that would have been excluded based on their Bonferroni adjustment, and furthermore, carefully demonstrated that some of the observed changes were quantitatively attributable to changes in cell type distribution as determined by cell surface markers. The gene expression profile had returned to baseline by the 24-h recovery time point, as no significant differences in gene expression from pre-exercise baseline were detected at that time point.

A substantial fraction of the 29 genes affected by exercise in whole blood in the study by Zieker et al. were similarly affected by heat shock of PBMCs *in vitro*. A cross-platform comparison analogous to the one described above for the Connolly study identified 35 sequences on the array used for *in vitro* heat shock (Sonna et al., 2002b) that corresponded to genes on the platform used by Zieker et al. Of these, about one third were similarly affected by *in vitro* heat shock and *in vivo* exercise (Table 3). This is comparable in magnitude to the previously noted overlap between heat shock

in vitro and the effects of exercise on the expression of "high interest" inflammatory response genes in the Connolly study (17–31%, see above).

The two exercise studies used markedly different array platforms, and we identified only eight genes affected by exercise in the Zieker study for which comparisons could be made between studies. The changes observed were more commonly discordant than concordant (i.e., increased in one study but decreased in the other or affected in the Zieker study but not in the Connolly study). The only concordance between the two datasets occurred between the immediate post-exercise sample obtained by Zieker et al. and the recovery time point obtained 1 h after the race by Connolly et al., time points that both occurred about the same time after the initiation of exercise (105 min in the Zieker study, 90 min in the Connolly study). At this time point, three genes showed concordant responses, namely, heat shock 27 kDa protein-1 (HSPB1) (increased in both studies), CD14 (increased in both studies), and interleukin 2 receptor beta (decreased in both studies). One important difference between the two studies was the ambient temperature at which subjects exercised. Whereas the Connolly study was performed in a laboratory setting at room temperature, the Zieker study was performed outdoors on a cool and humid day (ambient temperature 1°C). Also, the Connolly study examined gene expression in PBMCs, whereas the Zieker study used whole blood.

Despite the differences in methodology and findings between these two studies, the following general inferences can be made. First, acute physical exercise in fit males can produce changes in gene expression that are easily detectable in peripheral blood and circulating PBMCs using DNA microarrays and other technologies. Second, the changes in gene expression appear to be strongly time-dependent. Third, some of these changes in gene expression are likely due to exercise-induced changes in the distribution of PBMC subtypes whereas other changes cannot be explained solely by this phenomenon. Fourth, some of the PBMC gene expression changes include molecules that are inflammatory modulators, but not IL-6, which is consistent with the hypothesis that cells other than PBMCs (such as skeletal muscle) are the source of circulating IL-6 during exercise. Fifth, expression of at least some HSPs appears to be increased by exercise. Sixth, the data by Connolly et al. suggest that a large part of the PBMC response to exercise involves genes other than stress proteins and inflammatory modulators.

Some of the differences between *in vitro* and *in vivo* responses to heat stress can be accounted for by established mechanisms. For example, the IL1 receptor antagonist (IL1RN) was increased by exercise (e.g., the real-time PCR data in the Zieker study) but unaffected (1 sequence) or decreased (1 sequence) by heat shock *in vitro*. It has been postulated that the increased expression of IL1RN that occurs during exercise is induced by IL6 released by tissues such as skeletal muscle (Petersen and Pedersen, 2005). By contrast, increased expression of IL6 is not thought to occur in PBMCs during exercise (Connolly et al., 2004; Zieker et al., 2005) and *in vitro*, heat shock actually decreased IL6 mRNA levels in PBMCs (geometric mean expression ratio 0.37, 95% CI 0.16–0.85) (Sonna et al., 2002b). Thus, the discrepancies between the *in vitro* and *in vivo* IL1RN expression responses can be accounted for by the differences in availability of IL6.

Another informative difference between *in vitro* and *in vivo* responses is exemplified by CD14. Expression of CD14 mRNA was increased by exercise *in vivo* in both studies but was strongly decreased by heat shock *in vitro*. Interestingly, CD14 is a receptor for both bacterial LPS and apoptotic cells (Gupta et al., 1996; Devitt et al., 1998), and there is evidence in the literature that heat stress *in vivo* can lead to entry of LPS into the systemic circulation, putatively originating from the bowel (Hall et al., 2001; Lambert et al., 2002; Lambert, 2004). As with IL1RN, the discrepancy suggests the presence of a stimulus other than heat that modulates CD14 expression *in vivo*, and indeed, the observed change in CD14 in the Zieker study can be well accounted for by the observed changes in cell type distribution (Zieker et al., 2005).

The comparison of expression responses of PBMCs *in vitro* and *in vivo* suggest that exercise is a complex stimulus in which systemic and local factors can modify or override the effects of

hyperthermia on PBMC gene expression that would be expected based on the *in vitro* data. Responses that are similar between the two experimental systems can be accounted for, at least in part, by postulating a direct effect of hyperthermia on gene expression. By contrast, responses that differ between the two systems can lead to novel mechanistic hypotheses and insights, such as a search for novel mediators of responses to exercise, or can reaffirm the mechanistic importance of modifiers that have previously been identified (such as IL6). The concept that these complex responses can be dissected into component parts is perhaps the more important one, as it provides the intellectual basis for studies that compare gene expression responses that are beneficial (such as those involved in training and acclimatization) to those that are harmful (such as ones that contribute to dysfunction in EHI). Comparison of the component responses of beneficial and pathological responses may help identify candidate pathways for experimental and therapeutic manipulation.

Gene expression changes in PBMCs caused by exertional heat injury

Given the observation that PBMCs exhibit large gene expression responses to heat shock *in vitro* and to acute bouts of exercise *in vivo*, it is not surprising that extensive changes have been reported in these cells in the context of EHI. Comparisons of the PBMC expression responses to different stressors are informative, in that they can help identify candidate genes that might serve as markers of EHI as well as others that might be involved mechanistically in the disease processes.

Gene expression responses in EHI: similarity and differences to acute physical exercise

The effects of EHI on gene expression by PBMCs were recently reported in a study of Marine Corps recruits (Sonna et al., 2004). Samples were obtained from four subjects who presented to the medical clinic for emergency treatment of exertional heat injury. Samples were obtained at the time of presentation, 3 h after active cooling, and at a 24–48 h follow-up visit. The subjects had documented hyperthermia with evidence of organ injury (such as elevations in serum liver enzymes and/or creatine kinase), and had experienced prodromal symptoms suggestive of viral illness in the days preceding the onset of EHI. None met neurological criteria for the diagnosis of heat stroke. Control samples were obtained from three recruits several days before and several days after an intense field training exercise in hot weather. Gene expression analysis was performed on pooled samples using single-dye oligonucleotide arrays (Affymetrix U95Av2).

The results showed that the subjects experiencing EHI exhibited a time-dependent change in PBMC gene expression. The expression response was large, with 361 sequences showing increased expression at one or more of the time points studied and 331 showing decreased expression (the total expression response was slightly smaller than the 692 predicted by summing the two numbers, as some sequences showed increases at some time points and decreases at others). Many of the gene expression changes could be accounted for by heat shock responses previously documented in PBMCs *in vitro*, most notably among the HSPs. Others, however, could not be readily explained by the *in vitro* data. Importantly, and unlike the *in vitro* responses of PBMCs, about one fourth of the sequences most highly upregulated in EHI corresponded to interferon-inducible genes that are induced by interferons -alpha, -gamma, or both.

It is informative to compare the results of the Marine Corps recruit study to the changes reported by Connolly et al. (2004) (Tables 5A–C). Among the sequences whose expression was increased by exertional heat injury, approximately 1/6 were also increased by acute physical exercise, and many of these were also increased by heat shock *in vitro*. However, few of the highly upregulated EHI genes that were comparably affected by physical exercise were also interferon-inducible (Table 5A). By contrast, sequences that were highly upregulated by EHI but not by acute physical exercise commonly included interferon-inducible genes (Table 5B). A concept of component responses therefore emerges. Namely, EHI

and exercise share an expression response that can be ascribed to thermal stress *per se*, and the expression signature of EHI includes a component response that is functionally related to interferons but that is not present in acute physical exercise or in *in vitro* models of heat shock.

The dissection of gene signature responses into distinct components has important implications for improving the application of gene expression studies to the study of physiological states, as linking component responses to well-described functional outcomes allows correlations of molecular changes with the clinical and biochemical features of the disease. For example, increases in circulating interferons alpha and gamma do not appear to be part of the normal response to exhaustive exercise (Suzuki et al., 2002). In one case, a study of 16 elite marathon runners (race times less than 2 h and 38 min) found no significant differences in circulating interferons alpha and gamma levels before and after a marathon; by contrast, these subjects had marked elevations of IL-6 and IL-1 receptor antagonist, as expected (Suzuki et al., 2000). Likewise, neither the Connolly study nor the Zieker study reported an increase in mRNA encoding interferons alpha or gamma. By contrast, in the EHI study, a sequence corresponding to interferon gamma was significantly and strongly increased in EHI but was excluded from the final list of affected genes because of the strict post-hoc filter criteria used in that study (specifically, expression calls). Thus, these comparisons provide support for the hypothesis that interferons might play a role in the pathophysiology of some cases of human exertional heat injury.

Among sequences that exhibited decreased expression, little overlap occurred between EHI and acute physical exercise. Indeed, the number of overlapping downregulated sequences was far smaller than the number of overlapping upregulated sequences, representing only approximately 2% of all genes that were significantly decreased by EHI (as compared to ~1/6 of upregulated sequences, see above). The observation that the patterns of genes downregulation differ to such a great extent between the two conditions suggests the possibility that they might be used as biological markers to help distinguish between the two states.

These differences notwithstanding, there was nonetheless some overlap observed in the sequences that were downregulated by EHI and those downregulated by acute physical exercise. For example, both EHI and acute physical exercise resulted in decreased expression of the transcription factors *jun* and *myc*, which are known to have broad downstream effects. These shared changes in expression might represent normal responses to exercise that also occur in EHI, and might even be physiologically important. Interestingly, in tissue culture, decreased expression of *myc* has been shown to be important for cell survival after heat shock (Wennborg et al., 1995).

Because of the apparent interferon-inducible response observed among the upregulated genes in the EHI study, we re-examined the list of immune function genes that were downregulated by EHI. Interestingly, about half of these 43 downregulated sequences corresponded to genes that are normally expressed by T-cells (particularly activated and cytotoxic T-cells) and/or natural killer (NK) cells. Corresponding sequences in the Connolly study were found for 12 of these 43 sequences and of these, only 3 (2 of which were T-cell/NK cell related sequences) showed decreased expression at one of the time points examined; the remainder were upregulated. This observation gives rise to the hypothesis that EHI may produce changes in PBMC cell type distribution (specifically, of T-cells and/or NK cells) that are not part of the normal response to exercise.

Finally, several sequences were identified that were increased in response to exercise but that failed to show a significant expression response to EHI (Table 5C). This included genes that have broad effects on cellular function, such as NR4A2, CREM, EGR1, DUSP2, and DUSP3. Some of these were similarly affected by heat shock *in vitro*. One possible explanation for the finding of genes that are upregulated by heat shock *in vitro* and by acute physical exercise *in vivo* but not by EHI is the presence of signals during EHI that antagonize components of PBMC hyperthermia-induced responses. The identification of genes that distinguish normal exercise responses from those that characterize heat injury provides a rational basis for studies that explore their role as part of

Table 5. Gene expression responses of exercise, exertional heat injury, and heat shock

Sequence	Name	Interferon-responsive gene?	Expression ratio (EHI/control)			Effect of acute exercise		
			At presentation	After cooling	At follow-up	End-exercise/pre	Recovery/pre	Effect of heat shock *in vitro*
A: Examples of genes similarly affected by exercise and exertional heat injury								
Stress response genes and HSPs								
Increased								
HSPA1B/A	Heat shock 70 kDa protein 1B; HSP 70-2		32	18.7	1.1 (NS)	Increased	Increased	Up
HSPA1A	Heat shock 70 kDa protein 1A; HSP 70-1		31.4	22.3	1.2	NS or Increased	Increased	Up
HSPB1	Heat shock 27 kDa protein 1; HSP 28; HSP 27-1		9.6	32.4	1.1 (NS)	NS	Increased	Up
DNSJB1	DnaJ (Hsp 40) homolog, subfamily B, member 1; Hsp 40		8.4	1.5 (NS)	0.71 (NS)	Increased	NS	Up
HSPCA	Heat shock 90 kDa protein 1, alpha; HSP 90-1, alpha; HSP 90A		5.8	6.8	1.1 (NS)	NS	Increased	Up
DNAJA1	DnaJ (Hsp 40) homolog, subfamily A, member 1; HSPF4		3.5	1.4 (NS)	1.2 (NS)	Increased	NS	Up
Immune function								
Increased								
GZMB	Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)		5.6	1.8 (NS)	2.3	Increased	NS	Down
KIR3DL1	Killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 2		3.5	1.6 (NS)	0.58 (NS)	Increased	NS	NS
CTLA1	Similar to granzyme B (granzyme 2, cytotoxic T lymphocyte-associated serine esterase 1) (*H. sapiens*)		3.1	0.85 (NS)	2.0	Increased	NS	NS
Decreased								
IGHM	Immunoglobulin heavy constant mu		0.34	0.44	0.35	Decreased	NS	Down
Transcription								
Decreased								
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)		0.19	0.50	0.58 (NS)	Decreased	NS	NS
JUN	v-jun sarcoma virus 17 oncogene homolog (avian)		0.80	0.33	0.54	NS	Decreased	Up

Other							
Increased							
PLSCR1	Phospholipid scramblase 1	4.5	5.3	4.3	NS	Increased	NS
BCL2A1	BCL2-related protein A1	2.7	4.7	2	NS	Increased	NS
CCR1	Chemokine (C–C motif) receptor 1	1.9	4.5	3.3	NS	Increased	Down
S100A8	S100 calcium binding protein A8 (calgranulin A)	1.6	3.3	2.9	NS	Increased	NS
S100A9	S100 calcium binding protein A9 (calgranulin B)	1.7	3.4	2.6	NS	Increased	Up
S100A12	S100 calcium binding protein A12 (calgranulin C)	1.2 (NS)	3.3	1.8 (NS)	NS	Increased	NS
Decreased							
TBC1D4	TBC1 domain family, member 4	0.073	0.32	0.48 (NS)	Decreased	NS	NS

Sequence	Name	Interferon-responsive gene?	Expression ratio (EHI/Control)			Effect of heat shock *in vitro*
			At presentation	After cooling	At follow-up	
B: Examples of genes affected by exertional heat injury but not by exercise						
Upregulated sequences						
Stress response genes and HSPs						
HSPA6	HSP 70B′		24.7	3.6	3.8	Up
HSPA6	HSP 70B′		19.8	3.8	2.3	Up
SERPINH1	Serine (or cysteine) proteinase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1); HSP 47; colligin-1; colligin 2; SERPINH2		6.6	7.4	0.66 (NS)	Up
Immune function						
RSAD2	Radical S-adenosyl methionine domain containing 2′ cig5; Viperin	Yes	9.9	19.8	20.0	NS
IFIT3/IFIT4	Interferon-induced protein with tetratricopeptide repeats 3; interferon-induced protein 60; IFI 60; interferon-induced protein with tetratricopeptide repeats 4	Yes	9.1	15.6	19.4	Down
IFIT2	Interferon-induced protein with tetratricopeptide repeats; 2G10P2; cig42; IFI-54; GARG-39; ISG-54K	Yes	6.8	8.5	9.2	Down
OASL	2′–5′-oligoadenylate synthetase-like; thyroid hormone receptor interactor 14; TRIP14; p59OASL	Yes	6.1	9.5	8.5	NS
IFI27	Interferon alpha-inducible protein 27; p27; ISG12	Yes	2.8	39.8	20.7	NS

Table 5 (*continued*)

Sequence	Name	Interferon-responsive gene?	Expression ratio (EHI/Control)			Effect of heat shock *in vitro*
			At presentation	After cooling	At follow-up	
FCGR1A	Fc fragment of IgG, high affinity Ia, receptor for (CD64); CD64; Fc-gamma receptor I A1		3.4	10.9	8.6	NS
C3AR1	Complement component 3a receptor 1		3.3	8.6	3.0	Down
Other						
HISTH2H2AA	Histone 2, H2aa; H2AFO; H2A histone family, member O		10.7	22.3	17.6	NS
IFI44L	Interferon-induced protein 44-like; C1orf29; chromosome 1 open reading frame 29	Possibly	6.2	7.8	5.7	NS
SLC15A1	Solute carrier family 15 (oligopeptide transporter), member 1; peptide transporter HPEPT1; PEPT1	No[a]	5.8	1.0 (NS)	4.5	NS
PPP2R1B	Protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), beta isoform		2.3	8.2	12.2	NS
Downregulated sequences						
Cell growth, proliferation, and differentiation						
GOS2	Putative lymphocyte G_0/G_1 switch gene		0.033	1.4 (NS)	0.018	Down
HIPK3	Homeodomain interacting protein kinase 3		0.26	0.34	0.66 (NS)	NS
JAG1	Jagged 1 (Alagille syndrome)		0.75 (NS)	0.10	1.9	NS
Immune function						
FCER1A	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide		0.039	0.13	0.21	NS
CD96	CD96 antigen; TACTILE; T-cell activated increased late expression		0.11	0.12	0.041	Down
MAL	mal, T-cell differentiation protein		0.14	0.29	0.22	Up
Signal transduction						
IBTK	Inhibitor of Burton's tyrosine kinase		0.25	0.62 (NS)	0.43	Down
ARHH	Ras homolog gene family, member H		0.54 (NS)	0.19	0.48	Down
Transcription						
DRAP1	DR1-associated protein 1 (negative cofactor 2 alpha); Dr1-associated corepressor, mRNA sequence		0.13	0.39	0.15	NS

ZNF85	Zinc finger protein 85	0.89 (NS)	0.077	0.61 (NS)	NS
HMGA1	High mobility group AT-hook 1	0.57 (NS)	0.21	0.45	NS
Miscellaneous					
MS4A1	Membrane-spanning 4-domains, subfamily A, member 1; CD20 antigen	0.090	0.37	0.52 (NS)	NS
NUFIP1	Nuclear fragile X mental retardation protein interacting protein 1	0.13	0.39	0.087	Up
AHSA2[b]	Activator of heat shock 90 kDa protein ATPase homolog 2 (yeast)	0.26	0.31	0.56	Up
AGL	Amylo-1, 6-glucosidase, 4-alpha-glucanotransferase (glycogen debranching enzyme, glycogen storage disease type III)	0.39	0.20	0.34	NS
GAD1	Glutamate decarboxylase 1 (brain, 67 kDa)	0.40 (NS)	0.088	0.44 (NS)	NS
KIAA0982	KIAA0982 protein	0.61	0.16	0.69	Up
CENTD1	Centaurin, delta 1	0.80 (NS)	0.21	0.40	NS

Sequence	Name	Interferon-responsive gene?	Effect of acute exercise		Effect of heat shock *in vitro*
			End-exercise/pre	Recovery/pre	
C: Examples of genes affected by exercise but not by exertional heat injury					
Upregulated sequences					
Growth factors and transcription					
NR4A2	Nuclear receptor subfamily 4, group A, member 2		Increased	NS	Up
RGS1	Regulator of G-protein signaling-1		Increased	NS	Down
EREG	Epiregulin		Increased	NS	NS
CREM	cAMP responsive element modulator		Increased	Increased	NS
SAP30	Sin3-associated peptide, 30 kDa		NS	Increased	NS
EGR1	Early growth response 1		NS	Increased	Up
TLE3	Transducin-like enhancer of split 3 (E(sp1) homolog, *Drosophila*)		NS	Increased	NS
TGFB1	Transforming growth factor, beta-induced, 69 kDa		NS	Increased	NS
Immune function					
KIR2DL2	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 2		Increased	NS	NS
CPM	Carboxypeptidase M		NS	Increased	NS
CSF3R	Colony stimulating factor 3 receptor (granulocyte)		NS	Increased	Up
Stress response genes and HSPs					
DUSP2	Dual specificity phosphatase 2		Increased	NS	Up
DUSP3	Dual specificity phosphatase 3		NS	Increased	Up

Table 5 (*continued*)

Sequence	Name	Interferon-responsive gene?	Effect of acute exercise		Effect of heat shock *in vitro*
			End-exercise/pre	Recovery/pre	
Downregulated sequences					
LFNG	Lunatic fringe homolog (*Drosophila*)		Decreased	NS	NS
MEN1	Multiple endocrine neoplasia I		Decreased	NS	NS
TNFRSF12	Tumor necrosis factor receptor superfamily, member 12 (translocating chain association membrane protein)		Decreased	NS	Down
CD22	CD22 antigen		Decreased	Decreased	NS
NELL2	NEL-like 2 (chicken)		Decreased	NS	Up

Source: Data are from Connolly et al. (2004), Sonna et al. (2004), and Sonna et al. (2002b).

[a]The transport activity of this protein, however, is increased by IFN-gamma in some cell types (Buyse et al., 2003).

[b]Previously identified as KIAA0570/KIAA0729 based on UniGene clusters.

normal compensatory and even beneficial effects of exercise.

The following picture emerges from these comparative analyses of published studies is as follows. First, the gene expression signature of EHI can be subdivided into various identifiable components that appear to have mechanistic implications. One component includes genes that are similarly affected by heat shock *in vitro*, some of which are also part of the normal responses to exercise. These genes are good candidates for study as potential targets of heat-activated transcription factors, such as HSF-1. A second component includes genes that are similarly affected by exercise and EHI but that do not appear to be strictly dependent on hyperthermia for their change in expression. Changes in expression of these genes as a result of exercise would thus appear more likely to depend on factors other than heat *per se*. A third component involves genes that seem limited to EHI. This component includes several interferon-inducible genes that are upregulated in EHI as well as several genes that appear to be involved in T- and NK-cell function that are downregulated in EHI. Additional, better comparisons involving additional environmental exposures and time-dependent changes are likely to lead to the identification of additional informative component responses.

Additional conclusions that can be drawn from the comparative analysis presented include the following. First, the overlap between EHI and normal exercise responses may be much greater for sequences that show increases in expression than for sequences that show decreases in expression. These differences in the patterns of downregulation might, therefore, eventually prove useful as markers for injury. Second, finding genes that are affected by acute physical exercise but not EHI suggests that some of the changes characteristic of EHI might represent a failure to alter the expression of important genes that play a compensatory or protective role in normal exercise. Last, and most importantly, some of the differences in expression detected in these studies may be attributable not to changes in genes expression within a particular cell type, but rather to differences in the composition of circulating PBMCs (i.e., a change in the relative distributions of PBMC subtypes). For example, some of the downregulated genes might be accounted for by selective loss of certain subsets of T- and NK-cells in the peripheral circulation.

Inflammatory mediators, lymphocyte function, and exertional heat illness

The Marine Corps recruit study of gene expression responses to EHI does not permit a firm distinction to be made between changes that contribute to the pathophysiology EHI, those which are consequences of EHI (and as such, markers of the condition), and those which are epiphenomena. As commonly occurs in clinical studies, the Marine Corps recruit study did have methodological limitations that must be kept in mind when attempting to generalize the findings. These limitations include a low number of subjects with varying degrees of severity of EHI, use of pooled samples, and use of samples from control subjects that were drawn at different times from the index cases and at rest, rather than shortly after exhaustive exercise. There also may have been an artifact of population sampling given the small size of the study.

These limitations notwithstanding, two lines of evidence support the findings of the Marine Corps recruit study that interferons and other pro-inflammatory proteins might play a mechanistic role in some cases of EHI. The first is a series of *in vitro* studies that have reported that the response of cultured cells to heat shock is substantially altered by previous exposure history. For example, pre-exposure of porcine endothelial cells to LPS- or to TNF-alpha leads to a dose-dependent increase in cell death by apoptosis after heat shock (Buchman et al., 1993). A similar phenomenon was observed in a transformed murine cell line after exposure to interferon gamma (Abello and Buchman, 1994). These outcomes contrast with the normal response of these cells to heat shock in the absence of pre-exposure, which is survival and adaptation. The *in vitro* data thus suggest that delivery of a heat shock in the context of a pre-existing pro-inflammatory stimulus can lead to cell death by apoptosis, rather than survival and recovery.

The mechanism by which pro-inflammatory stimuli, such as LPS and TNF-alpha, can lead to decreased cellular survival after heat shock might involve transcription factor NF-kappa-B (DeMeester et al., 2001). Normally, NF-kappa-B exists in an inactivated state in the cytoplasm, bound to the inhibitor I-kappa-B (Malhotra and Wong, 2002). Activation of NF-kappa-B by pro-inflammatory stimuli involves phosphorylation of this inhibitor by I-kappa-B kinase (IKK), which in turn to the ubiquitinylation and proteasome-mediated degradation of I-kappa-B, translocation of NF-kappa-B to the nucleus, and activation of NF-kappaB — dependent transcription (Curry et al., 1999; Shanley et al., 2000; Yoo et al., 2000). Heat shock inhibits the activation of NF-kappa-B through a variety of mechanisms, of which the most important is believed to be inhibition of IKK activity, which in turn prevents phosphorylation of I-kappa-B and its dissociation from NF-kappa-B (Wong et al., 1997; Scarim et al., 1998; Curry et al., 1999; Shanley et al., 2000; Yoo et al., 2000). Increases in I-kappa-B expression have also been observed as a result of heat shock (Wong et al., 1999; Pritts et al., 2000). In the presence of pre-existing pro-inflammatory stimuli, which activate NF-kappa-B, the subsequent delivery of a heat shock appears to be sufficient to shift cellular fate toward apoptosis (DeMeester et al., 2001).

As noted previously, although exertional heat illness can result from a single exposure to heat stress that overwhelms available compensatory responses, epidemiological data suggest that for some individuals, exertional heat illness may occur as the result of a "two-hit" process. These subjects present with exertional heat illness at ambient temperatures and exercise intensities that would normally be tolerated (Kark et al., 1996; Epstein et al., 1999; Gardner and Kark, 2001), commonly report feeling ill in the days preceding exertional heat illness (Shibolet et al., 1967; Epstein et al., 1999), and frequently report prior day exposure to heat (Kark et al., 1996). It thus seems plausible that some precedent pro-inflammatory exposure (previous heat injury or viral illness) could make a person more susceptible to exertional heat illness during a subsequent unremarkable exercise-heat exposure. The initial exposure might act to augment the hyperthermia of exercise, make tissue more susceptible to injury for a given heat stress, or both.

That interferons could provide such a first hit stimulus is supported by a case series in which circulating levels of interferon gamma were measured in patients who presented to the emergency department with heat stroke (Bouchama et al., 1993). Of 10 patients for which interferon levels were available, half showed elevated levels of circulating interferon gamma that generally diminished after treatment. As noted (previous section), increased expression of interferons alpha and gamma are not generally considered a normal feature of acute physical exercise. Thus, the observation of increases in circulating interferon gamma in heat stroke suggests that, at least in some cases of human heat illness, interferons might play a role in the pathophysiology of the disease. Although the observational data cannot distinguish between a causal role for interferon gamma and the possibility that it is merely a consequence of heat illness, when coupled with the observed responses of animal cells *in vitro*, it seems at least plausible to suggest that a pre-existing stressor that increases circulating levels of interferon gamma (or alpha) might presensitize cells and tissues to heat stress in a manner that produces morbidity under heat loads that might otherwise be well tolerated. Further observational and experimental works are needed to test this hypothesis.

Finally, in addition to increases in the expression of genes that respond to inflammatory mediators, the Marine Corps recruit study identified a component of altered T- and NK-cell gene expression in subjects with EHI. This finding suggests that EHI may involve protein-driven changes that involve cellular immunity. Understanding the intersection between thermal stress and immune function may thus expand and redirect our thinking about thermal biology.

Summary

Gene expression signature data to date shows that EHI produces time-dependent changes in expression that are easily detectable in PBMCs. These changes can be dissected into component parts

that include effects resulting for thermal stress, exercise, and those that likely reflect alterations in immune function. Cross-platform comparisons such as those outlined in this chapter have significant limitations that do not prevent the identification of major component responses, but do limit the confidence with which many individual sequences can be implicated.

Exertional heat illnesses occur when heat load exceeds heat dissipation beyond the point that the individual can physiologically tolerate. Thermal biology research cannot alter that fact that every individual has a heat load at which function is no longer possible. It can, however, define the mechanisms that allow for more efficient heat dissipation, those that allow more effective adaptation to a given heat load, and those that alter the point at which an individual becomes sick. Conceptually, cellular responses to a heat load can be dichotomized into survival and adaptation, which typically permit continued effective function at a new (and higher) thermal steady state, and dysfunction, which tend toward cell death (by apoptosis if possible, necrosis if not). The factors that determine which pathways will predominate and the mediators on which they depend are now being elucidated, in part by comparison of pathway-specific components of gene expression signature and associations with the clinical and biochemical profiles of heat related injury.

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The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, or decision, unless so designated by other official documentation. Approved for public release; distribution unlimited.

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